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Treatment of Reperfusion Injury With Intracoronary Calcium Channel Antagonists and Reduced Coronary Free Calcium Concentration in Regionally Ischemic, Reperfused Porcine Hearts

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The effect of intracoronary diltiazem, EGTA (ethylene-bis-(β-aminomethylether)-N,N′-tetraacetic acid), nifedipine, verapamil and isotonic saline solution as placebo on reperfusion injury was investigated in regionally ischemic, reperfused porcine hearts. The left anterior descending coronary artery was distally occluded for 45 min and was reperfused for 3 days. Intracoronary infusion was started immediately before reperfusion and continued during 45 min of reperfusion. Infarct size was determined as the ratio of infarcted (tetrazolium stain) to ischemic myocardium (dye technique). Regional systolic shortening was assessed by sonomicrometry.

Apart from left ventricular end-diastolic pressure before ischemia and during 45 min of reperfusion, global hemodynamic values in the five treatment groups did not differ; in particular, calculated left ventricular oxygen consumption before and during ischemia was equally low. Intracoronary EGTA decreased coronary venous free calcium concentration to about 70% of baseline value. Infarct size was reduced from 76 ± 10% (control group, n = 8) to 60 ± 10% ($p < 0.01$) by intracoronary diltiazem (n = 8) and to 55 ± 15% ($p < 0.01$) by intracoronary EGTA (n = 8). Insignificant reductions in infarct size were found after treatment with intracoronary verapamil (63 ± 18%, n = 8) and intracoronary nifedipine (68 ± 9%, n = 7). Regional systolic shortening of the risk region, which did not differ among the groups before occlusion and during ischemia, recovered to the greatest extent in the EGTA-treated pigs ($p < 0.01$ compared with values in the control group). Treatment with intracoronary calcium antagonists resulted in only marginal improvement of systolic shortening. Reperfusion-induced rhythm disturbances were not beneficially affected by either treatment.

It is concluded that reperfusion injury exists in regionally ischemic, reperfused porcine hearts, which can be attenuated by intracoronary EGTA and intracoronary diltiazem administered during the first 45 min of reperfusion. The favorable action of EGTA is ascribed to a reduced ionized extracellular calcium concentration, whereas the beneficial mechanism of diltiazem is unknown.

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Early restoration of blood flow represents a potent means to reduce infarct size and mortality in patients with acute myocardial infarction (1,2). Whether reperfusion of ischemic myocardium may cause cell death in myocytes that have survived the phase of ischemia is still subject to controversy. Treatments aimed at suppressing biochemical reactions that are considered to be involved in the genesis of reperfusion injury in intact animals, e.g., scavenging of oxygen free radicals (3–6) or anti-inflammatory agents (7–9), have exhibited conflicting results. Sudden intracellular uptake of calcium during reperfusion (10), which can result in contraction band necrosis and mitochondrial accumulation of calcium (11), appears to be another mechanism for the evolution of reperfusion injury. In this study we examined whether intracoronary treatment with three calcium channel antagonists administered only during reperfusion, and lowered intracoronary free calcium concentration during reperfusion, would reduce infarct size and improve regional systolic shortening in porcine hearts subjected to 45 min of ischemia and 3 days of reperfusion.

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Methods

Medication, anesthesia, general surgical procedures and measurement of infarct size have been described in detail in previous studies (6,9,12). Fifty male and female farm pigs weighing 35 to 48 kg were used in this study.

General experimental design. After median thoracotomy, the left anterior descending coronary artery was dissected free at the beginning of its distal third. Left ventricular pressure and its first derivative (dP/dt max) were measured with a 5F Millar catheter-tipped manometer. Blood pressure was assessed in the ascending aorta with a fluid-filled catheter connected to a Statham transducer. A 5F multipurpose catheter was advanced from a jugular vein through the coronary sinus into the ostium of the great cardiac vein. A 4F Rentrop catheter with a 3F tip (USCI) was introduced under fluoroscopic control through the right carotid artery into the proximal part of the left anterior descending coronary artery. One pair of ultrasonic crystals (13) was implanted in the subendocardial layer of the planned ischemic region oriented parallel to the short axis (14).

Regional systolic shortening was determined by the ultrasonic transit time method (15,16). Analogue tracings of the signals were obtained from direct writing recordings. The end-diastolic distance of the crystals was determined at the onset of ventricular systole. Relative systolic shortening was assessed as the difference of distances between the beginning of ventricular systole and aortic valve closure over end-diastolic distance × 100. Regional systolic shortening and global hemodynamic variables, including left ventricular peak pressure, left ventricular end-diastolic pressure, diastolic blood pressure, dP/dt max and heart rate, were recorded before occlusion, at 5 min intervals during ischemia and during 45 min of reperfusion and after 3 days of reperfusion. Left ventricular oxygen consumption ($M\dot{V}O_2$) was calculated from the hemodynamic data before occlusion and during ischemia with use of Bretschneider's equation (17).

The coronary artery was occluded at the prepared site for 45 min and was reperfused for 3 days. In this preparation, occlusion of the coronary artery at the specified site renders about 15 g of the left ventricular muscle (average weight 120 g) ischemic. Forty-five minutes after the onset of reperfusion, the chest was closed in layers and the animal was allowed to recover. On the third postoperative day, the pig was reanesthetized and thoracotomy was repeated. After measurement of global hemodynamics and regional systolic shortening, the coronary artery was occluded at exactly the same site, a central venous injection of 20 ml 10% fluorescein sodium solution was administered to label the well perfused myocardium with a green fluorescent color and the heart was excised. The area at risk of necrosis was determined by planimetry of the nonfluorescent myocardium of four heart slices with use of enlarged photographs (13 × 18 cm). Infarcted myocardium was assessed by incubating the heart slices in nitroblue tetrazolium solution (18,19). Infarct size was calculated as a ratio of infarcted myocardium over risk region × 100.

Experimental protocol. Fifty pigs were randomly assigned to one of the four treatment groups or to the control group. The intracoronary infusion of the active compounds or of isotonic sodium chloride solution as placebo was started 60 s before release of the coronary occlusion and was continued for 45 min during reperfusion. The four treatment groups, each consisting of 10 pigs, received either intracoronary diltiazem (Gödecke) (0.25 mg/min for 16 min followed by 0.125 mg/min for 30 min), intracoronary nifedipine (Bayer) (0.05 mg/min for 16 min followed by 0.025 mg/min for 30 min), intracoronary verapamil (Knoll) (0.125 mg/min for 16 min followed by 0.062 mg/min for 30 min) or intracoronary EGTA (ethylene-bis-(β-aminomethylether)-N,N′-tetraacetic acid; analytical grade; Serva) neutralized to a pH value of 7.4 with sodium hydroxide. The dosage of EGTA was adjusted according to systolic blood pressure and ranged between 20 and 32 μmol/min. In general, the initial dose was 28 μmol EGTA/min, and the concentration was reduced when systolic blood pressure decreased to <70 mm Hg.

Coronary venous blood concentrations of total and free calcium were determined with standard laboratory techniques before occlusion and after 2, 5, 10, 20 and 40 min of reperfusion. Arterial calcium concentrations were measured before and after 40 min of reperfusion.

Statistics. All data are presented as mean values ± SD. Overall statistical comparisons among the groups were performed with the Kruskal-Wallis H test. When this test indicated statistical significance, the results of the different groups were analyzed with the Wilcoxon-Mann-Whitney *U* test (20). Results within a group were compared with the Wilcoxon test for paired observations. Probability values <5% ($p < 0.05$) were considered statistically significant.

Results

During the first postoperative night, premature deaths occurred in 10 pigs—2 each in the diltiazem, verapamil and control groups, respectively, 3 in the nifedipine group and 1 in the EGTA group. In the latter group, one pig that developed ventricular fibrillation on reperfusion could not be resuscitated. Therefore, the results are based on 7 experiments in the nifedipine group and on 8 in each of the other four groups (total 39 pigs).

Rhythm disturbances. Ventricular fibrillation during ischemia occurred in 15 pigs: 2 in the diltiazem group, 3 each in the nifedipine, verapamil and control groups and 4 in the EGTA group. In most experiments, reperfusion induced various types of tachyarrhythmias, including accelerated idioventricular rhythms, premature ventricular complexes,

Table 1. Global Hemodynamic Variables In the Five Treatment Groups

Group	Before Occlusion	During Ischemia	Reperfusion	
			45 min	3 days
LVPP (mm Hg)				
D	99 ± 14	98 ± 13	91 ± 6	91 ± 17
E	93 ± 11	90 ± 12	79 ± 11*	82 ± 17
N	105 ± 18	100 ± 12	92 ± 9	101 ± 17
V	104 ± 15	100 ± 7	81 ± 9*	105 ± 18
C	107 ± 14	94 ± 15*	86 ± 13*	104 ± 6
LVEDP (mm Hg)				
D	13 ± 6	13 ± 5	12 ± 7	11 ± 4
E	6 ± 2	9 ± 2*	8 ± 3	10 ± 3
N	12 ± 6	15 ± 5	16 ± 5	13 ± 6
V	9 ± 4	9 ± 3	8 ± 4	10 ± 4
C	11 ± 5	10 ± 6	8 ± 3	12 ± 4
DBP (mm Hg)				
D	61 ± 15	64 ± 13	61 ± 9	61 ± 18
E	62 ± 10	60 ± 11	58 ± 9	51 ± 19
N	72 ± 20	71 ± 13	66 ± 9	70 ± 12
V	66 ± 9	66 ± 10	56 ± 11*	71 ± 18
C	74 ± 18	68 ± 14	64 ± 11	70 ± 10
Heart rate (beats × min^{-1})				
D	77 ± 25	86 ± 20*	106 ± 10*	91 ± 8
E	72 ± 9	74 ± 8	108 ± 14*	82 ± 17
N	72 ± 20	76 ± 14	106 ± 16*	92 ± 8*
V	61 ± 7	71 ± 10	102 ± 19*	90 ± 9*
C	70 ± 17	88 ± 14*	118 ± 14*	105 ± 14*
dP/dt max (mm Hg × sec^{-1})				
D	1,738 ± 534	1,720 ± 478	1,686 ± 450	1,162 ± 177*
E	1,775 ± 349	1,567 ± 218	1,283 ± 306*	1,088 ± 295*
N	2,028 ± 756	1,759 ± 510	1,745 ± 682	1,429 ± 330
V	1,650 ± 421	1,689 ± 468	1,377 ± 376*	1,200 ± 177*
C	1,662 ± 496	1,638 ± 583	1,639 ± 361	1,488 ± 461
$M\dot{V}O_2$ (ml × min^{-1} × 100 g^{-1})				
D	5.9 ± 1	6.3 ± 0.8*		
E	5.7 ± 0.7	5.6 ± 0.7		
N	5.9 ± 1.8	5.8 ± 0.8		
V	5.3 ± 0.5	5.7 ± 0.8		
C	5.6 ± 1.2	5.9 ± 0.8		

*Significantly different compared with preischemic value. Values are mean ± SD. C = control; D = diltiazem; DBP = diastolic blood pressure; E = EGTA (ethylene-bis-(β-aminoethylether)-N,N'-tetraacetic acid); EDP = end-diastolic pressure; LV = left ventricular; $M\dot{V}O_2$ = myocardial oxygen consumption; N = nifedipine; pp = peak systolic pressure; V = verapamil.

short runs of ventricular tachycardia and ventricular fibrillation. Ventricular fibrillation on reperfusion was observed in three pigs in the EGTA group, two in the verapamil group and one in the diltiazem group. It did not occur in the nifedipine-treated or control groups. Except for one pig in the EGTA group, ventricular fibrillation was always immediately reversed by direct electrical countershocks.

Reperfusion-induced rhythm disturbances were characterized by a sudden increase in heart rate. Comparison of the heart rates measured immediately before and after 1, 2, 3, 4, 5 and 10 min of reperfusion did not indicate any beneficial effect of the four treatments on reperfusion-induced tachyarrhythmias. The mean increases in heart rate were 24% (diltiazem group), 73% (EGTA group), 38% (nifedipine group), 37% (verapamil group) and 21% (control group).

Global hemodynamics (Table 1). Significant differences among the groups (Kruskal-Wallis test) were observed only for left ventricular end-diastolic pressure before occlusion and during 45 min of reperfusion. Before occlusion, left ventricular end-diastolic pressure was significantly lower in the EGTA group than in the nifedipine ($p < 0.01$) or the diltiazem ($p < 0.01$) group. During 45 min of reperfusion, it was significantly higher in the nifedipine-treated pigs than in the pigs in the control ($p < 0.01$), EGTA ($p < 0.01$) and verapamil-treated ($p < 0.02$) groups. Calculated left ventric-

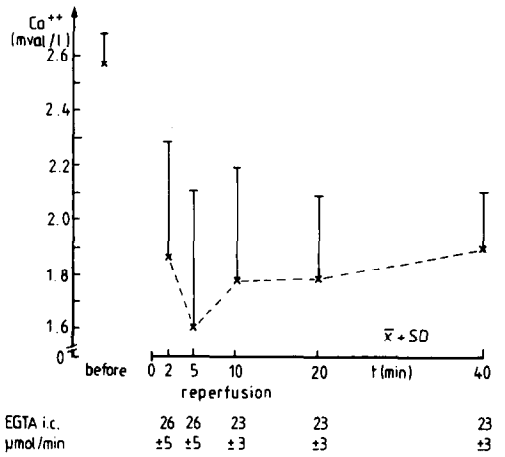


Figure 1. Free coronary venous calcium concentration in the EGTA-treated animals before treatment and during 40 min of reperfusion. The dosage of intracoronary (i.c.) EGTA (ethylene-bis-(β-aminoethylether)-N,N′-tetraacetic acid) is given at the bottom of the figure. Ca^{++} = calcium ion; mval = mmol/valence.

ular oxygen consumption before and during ischemia was equally low in all groups.

Effect of EGTA treatment on free coronary vein calcium concentration. Coronary vein calcium concentrations did not change significantly in calcium antagonist and control experiments during reperfusion. Total arterial and coronary vein calcium concentrations that amounted to 4.9 ± 0.3 mval/liter were not significantly affected by intracoronary EGTA treatment. Before occlusion, free coronary vein calcium concentrations were slightly (0.06 ± 0.03 mval/liter) but significantly higher than arterial concentrations. Intracoronary EGTA therapy reduced mean free coronary vein calcium concentrations to about 70% of baseline values (Fig. 1).

Infarct size. Risk regions of the five groups did not differ. The areas at risk of necrosis that were determined on photographs measured 980 ± 354 mm^2 (diltiazem group), 1,278 ± 442 mm^2 (EGTA group), 1,218 ± 336 mm^2 (nifedipine group), 1,043 ± 170 mm^2 (verapamil group) and 1,184 ± 246 mm^2 (control group). Infarct size in the five groups is depicted in Figure 2. Treatment with diltiazem and with EGTA significantly reduced mean necrosis formation by 21% ($p < 0.01$) and 28% ($p < 0.01$), respectively. Infarct size in these two groups was also significantly smaller compared with that in the nifedipine treatment group ($p < 0.05$). Reduction in infarct size by verapamil did not reach statistical significance, although mean infarct size in this group was only 5% larger than that in the group treated with diltiazem.

Recovery of regional systolic shortening. Baseline values of the end-diastolic distances of the ultrasonic crystals did not differ. These were 10.6 ± 2.8 mm (diltiazem group), 10.7 ± 1.8 mm (EGTA group), 9.8 ± 1.7 mm (nifedipine group), 9.5 ± 0.8 mm (verapamil group) and 10.4 ± 2.4 mm (control group). In all experiments, reperfusion induced a pronounced decrease in end-diastolic distance. After 10 min of reperfusion, mean end-diastolic distance was reduced to 75% (diltiazem group), 72% (EGTA group), 79% (nifedipine group), 74% (verapamil group) and 69% (control group) compared with the values immediately before reperfusion. Regional systolic shortening of the risk region in the five groups is shown in Figure 3. No difference among the groups was observed before ischemia and before reperfusion. After 3 days of reperfusion, regional systolic shortening had improved to the greatest extent in the EGTA-treated pigs ($p <$

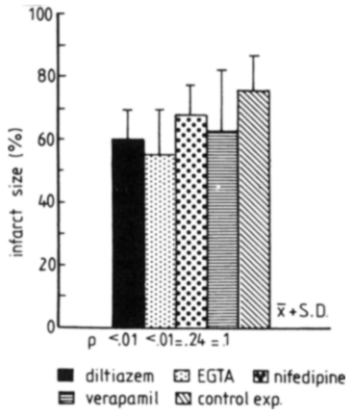
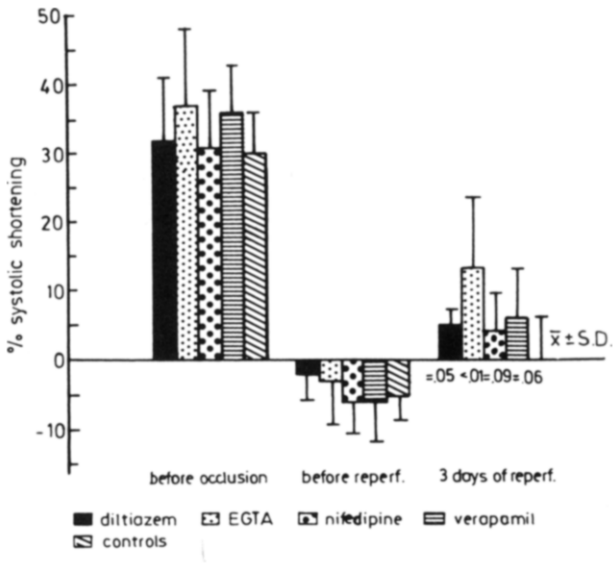


Figure 2. Infarct size of the five treatment groups after 45 min of ischemia and 3 days of reperfusion; p values are related to the infarct size of the control group. Other abbreviations as in Figure 1.

Figure 3. Regional systolic shortening of the risk region of the five groups; p values are related to regional systolic shortening of the control group after 3 days of reperfusion (reperf.). Other abbreviations as in Figure 1.



0.01 compared with control). Recovery of regional myocardial function of the diltiazem-, nifedipine- and verapamil-treated animals reached borderline significance.

Discussion

Calcium in regionally ischemic, reperfused myocardium. Myocardium reversibly injured by 10 min of transient ischemia does not accumulate calcium. However, 40 min of regional ischemia followed by reperfusion results in an 18-fold increase of calcium in the damaged tissue (21), which occurs during the early phase of reperfusion (22). Much of the intracellular calcium is found in dense bodies within mitochondria. The process of mitochondrial uptake of calcium does not indicate that these organelles have died already. On the contrary, intact mitochondria prefer to accumulate calcium in preference to phosphorylation of adenosine triphosphate (ATP) (23). Mitochondria overloaded with calcium exhibit profound functional deterioration. Oxidative phosphorylating activity and ATP-generating capacity become considerably impaired (23,24). Whether the intracellular uptake of calcium during reperfusion determines the transition from reversible to irreversible injury in some jeopardized myocytes is still unresolved. Various studies (25–27) in globally ischemic heart preparations demonstrated that functional recovery after transient ischemia can be improved by a reduced extracellular calcium concentration. On the other hand, elevated extracellular calcium concentrations abolished functional impairment observed after short-term ischemia in regionally ischemic, reperfused canine hearts (28). Reperfusion of regionally ischemic canine myocardium with calcium-free blood revealed contradictory results in regard to ultrastructural cell damage. It was reported in one study (29) that this treatment preserved morphology after 45 min of transient ischemia, whereas another study (30) that used a similar protocol failed to demonstrate this beneficial effect.

The results of the present study support the concept that reperfusion injury exists in regionally ischemic hearts. Reduced free coronary calcium concentrations during 45 min of reperfusion significantly decreased infarct size and improved recovery of regional systolic shortening. Although reduced afterload has been found to favorably affect systolic bulging during ischemia (31), it is unlikely that the lower left ventricular peak pressure in the EGTA group accounts for the better recovery of regional function after 3 days of reperfusion. Whether EGTA treatment only accelerated functional recovery or whether this therapy induces ultimate improvement of systolic shortening by reducing infarct size cannot be answered with certainty. Because we were unable to measure free calcium concentrations in the arterial blood that perfused the risk region, we had to rely on blood concentrations determined in the corresponding vein. Extrapolation of coronary vein to coronary artery free calcium concentration may result in a small error. Because free coronary vein calcium concentrations could not be measured on-line, it was impossible to achieve equal reductions of ionized calcium by the infusion of EGTA.

Administration of intracoronary EGTA is not without risks. Preliminary experiments had demonstrated that the therapeutic range of intracoronary EGTA is rather small. The intracoronary infusion of 40 μmol EGTA/min, which decreased free coronary vein calcium concentration to about 0.6 mval/liter, induced ventricular tachycardia, and higher intracoronary EGTA doses always resulted in ventricular fibrillation. The optimal treatment durations and dosages of intracoronary EGTA and of intracoronary calcium antagonists during reperfusion are not known. We treated the pigs during 45 min of reperfusion because it was demonstrated that 20 min of reperfusion was sufficient to reverse ultrastructural changes of reversibly injured myocytes (32).

Calcium antagonists for the treatment of reperfusion injury. The mechanisms involved in the cellular uptake of calcium during reperfusion are not clearly defined. It is suggested that calcium enters the myocyte primarily by an increased permeability of the sarcolemma to calcium, which does not require membrane disruption (33,34). Whether calcium may also enter the cells by a sodium-calcium exchange (35,36) or by the voltage-dependent slow calcium channel is subject to controversy. Furthermore, the role of a fast calcium channel (37,38) for the cellular uptake of calcium on reperfusion has not yet been studied. In an isolated septum preparation, neither verapamil (39) nor nifedipine (40) was able to prevent intracellular calcium accumulation when the drug was administered during reperfusion. However, diltiazem was partially protective in a papillary muscle preparation (41) and nifedipine delayed the intracellular uptake of calcium in isolated rat hearts (34).

At least 37 studies have been performed to test the effect of calcium antagonists on infarct size in different animal models (reviewed in Ref. 42). In three of them (43–45), a calcium antagonist was administered only during ischemia and during reperfusion, which lasted for 3 to 4 h (43,44) or 1 week (45). Infarcted myocardium was delineated by tetrazolium staining. In two of the studies (43,44), infarct size was insignificantly reduced by either intracoronary verapamil (43) or intravenous diltiazem (44). However, a reperfusion period of 3 to 4 h, which was used in these studies, may have been too short to identify accurately necrotic myocardium by a tetrazolium stain when areas of no reflow were present (19). In the third study (45), nisoldipine, a derivative of nifedipine, was ineffective in treating reperfusion injury in baboons. In that study and in the canine study (43) that tested verapamil, reperfusion was started only after 3 h of ischemia, a time when mitochondrial accumulation of calcium may no longer occur (11). In the present study, the dosages of the calcium antagonists were guided by therapeutic plasma levels of these compounds in humans (46). The

intracoronary infusion of the calcium antagonists warranted blood concentrations in the coronary artery that were ≥2.5- to 5-fold higher than usual therapeutic plasma levels, even when maximal coronary vasodilation was taken into account.

Comparison of the three calcium antagonists. The reason for the different effects of the three calcium antagonists on infarct size remains to be elucidated. Although the vasodilator adenosine administered during reperfusion has reduced infarct size and improved functional recovery in canine hearts (47), it is unlikely that the results of our study can be ascribed to different degrees of vasodilation because nifedipine is a potent vasodilator (48). The three calcium antagonists produced marginal improvement in regional systolic shortening. Whether this effect can be extrapolated to a better ultimate improvement of regional function due to infarct size reduction is uncertain because it has been demonstrated (49) that verapamil attenuated postischemic dysfunction in canine hearts after short-term ischemia when no infarctions were present.

Conclusions. Our study suggests that reperfusion injury exists in regionally ischemic porcine hearts and that this injury can be attenuated by intracoronary EGTA and intracoronary diltiazem administered during 45 min of reperfusion. At the given drug dosages, diltiazem was superior to nifedipine but only insignificantly better than verapamil.

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